SPORE FORMATION AMONG THE ANAEROBIC BACTERIA

I. THE FORMATION OF SPORES BY CLOSTRIDIUM SPOROGENES IN NUTRIENT AGAR MEDIA

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This investigation was initiated with the purpose of determining more precisely the conditions under which the clostridia produce spores. In this paper we shall present the data obtained in our initial approach to the problem. It must be realized, of course, that these preliminary results may hold only for a similar strain, which has the same immediate past as the organism used, and that other spore-forming bacteria may differ not only quantitatively in their response to various environments, but perhaps even more fundamentally, in the very nature of this response.

Considerable work has already been done on this problem. By far the larger part has been devoted to investigations of the physiology of spore formation among the aerobic bacteria rather than to anaerobic sporulation. Fairly comprehensive reviews on the formation of spores by the bacilli have been published by Brunstetter and Magoon, and by Cook. The opinion shared by many modern workers is that spores are formed more quickly and in greater numbers, by most aerobic bacteria, in media which contain less nutrient, than in media which offer an abundance of available nitrogen and carbon sources (Henrici, 1928; Williams, 1931; Brunstetter and Magoon, 1932; Tarr, 1933). There are, however, some very definite exceptions: for example, Bacillus fusiformis sporulates to a much greater extent in the more concentrated solutions of peptone (Brunstetter and Magoon, 1932).

In addition, the importance of other factors in the causation
of aerobic sporulation has frequently been asserted. Some bacilli form spores over a much narrower temperature and pH range than that in which growth occurs. The minimal oxygen tension for growth also is generally lower than that for the formation of spores (Brunstetter and Magoon, 1932). The influence of the salt balance of the medium may be considerable; univalent cations apparently "stimulate" the formation of spores, while polyvalent cations do not (Fabian and Bryan, 1933). Daranyi concluded that the decrease of water content which occurs naturally when the cell "ages" is of decisive importance in the induction of spore formation, and that the process might be accelerated by the dehydration of the cells (Cook, 1932). Finally, these various conditions surrounding the growth of the culture do not alone determine the degree and rate of spore formation; equally important determinants are the species and variety of bacterium used, and the previous history of the particular strain.

The formation of spores by anaerobic bacteria has been the subject of many investigations during the last half century, a large number of which, however, were initiated not as problems in bacterial physiology, but rather in the course of more utilitarian studies. Perhaps a majority of these papers were concerned with Clostridium welchii, the subject of whose spore-forming ability has been of considerable interest because of the difficulty with which the phenomenon can be controlled. The importance of the pH of the medium was early recognized (Schattenfroh and Grassberger, 1900). In 1910 Fitzgerald, a pupil of Noguchi, published the results of some of their experiments; she concluded that the addition of alkali to the original medium (N/200-N/50 NaOH) induced sporulation by Bacillus aerogenes capsulatus, while the presence of fermentable carbohydrates inhibited the process. Twenty years later, Torrey, Kahn, and Salinger demonstrated that (their strains of) C. welchii formed few or no spores in media more acid than a pH of 6.6. This fact is of interest in that people with gastric achyilia (a condition in which no HCl is secreted into the stomach) maintain a considerably higher pH in the upper and middle parts of the small intestine (pH 6.8–7.9).
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than do normal people (pH 4.1–6.7); and these people are found to harbor thousands of times the number of C. welchii spores that other people do.

The hydrogen ion concentration is a controlling factor in the sporulation of other anaerobes also. Thus, Möhrke found that in most media, Clostridium tetani, C. botulinum, C. putrificum, and C. septicum sporulate much more readily in neutral or slightly alkaline media than in those having a pH under 6.6. However, according to Leifson, C. tetani and C. botulinum have pH optima for spore formation at about 6.2. This discrepancy may be due partly to the use of different strains with different backgrounds, and partly to the high nutrient concentration of Leifson's medium (2 per cent agar, 1 per cent peptone, and 0.5 per cent meat extract). Generally, media rich in albuminous or other nitrogenous materials accelerate the rate of formation of spores among the anaerobes mentioned above, and in some instances may even mask the inhibitory effect of an acid pH (Möhrke, 1926).

The relationship of oxygen tension to the sporulation of anaerobes has not been comprehensively studied. The difficulties inherent in such an investigation are obviously great, and results have been published hitherto only on isolated or imperfectly controlled experiments. Some evidence has been brought forward that the infiltration of oxygen into an "anaerobic" medium does not necessarily inhibit the formation of spores. Matzschita found that exposure of actively growing cultures to oxygen resulted in an acceleration of the spore-forming process. Zinsser, a few years later, mentions that spores were found only in those cultures of C. tetani and Clostridium chauveii which were less completely freed of oxygen, under the conditions of the experiment. Smidt, in 1924, found that in broth cultures of Clostridium sporogenes which were aerated at intervals of 24 hours, "up to a period of 5 or 6 days sporulation appeared to be markedly increased as compared with similar cultures not aerated." Recently, Underkofler, Christensen, and Fulmer have reported that spore formation by Clostridium acetobutylicum was induced on sugar media (on which this organism usually does not sporulate because of the acidity produced), by spreading the already fer-
mented sugar medium in a thin layer in a large sterile flask; under such conditions and exposed to air, spores are readily formed. On the other hand, very different conclusions have also been published: Leifson, in 1931, stated that the optimum oxygen concentration for anaerobic sporulation was very close to zero. He found that both the degree of growth and the per cent of spore formation, for most anaerobes, was less on agar slants (of 2 per cent agar, 1 per cent peptone, and 0.5 per cent meat extract) under a tension of 1 cm. of oxygen than under as nearly anaerobic conditions as he could effect.

Lastly, the stimulatory effects on spore formation of various types of substances in the medium have been emphasized by some investigators. These substances fall into various categories. Thus, certain inorganic ions like the ammonium ion and the sulfate ion have been shown to stimulate the formation of spores by both _C. tetani_ and _C. botulinum_ (Leifson, 1931). We have already pointed out the accelerating effects of the addition of utilizable sources of nitrogen (e.g. albumin, serum, etc.) to the medium; strangely, several unfermented carbohydrates, and perhaps some fermentable polysaccharides (e.g. raffinose, starch) stimulate the formation of spores by _C. welchii_ (Schattenfroh and Grassberger, 1900; Fitzgerald, 1910; Svartz, 1934). The symbiotic stimulation of sporulation by _C. welchii_ by unknown fecal organisms (Simonds, 1915) or by _Bacillus faecalis alkaligenes_ (Shoetensack, 1932) may possibly be due to the accumulation of certain products of the symbiont’s metabolism. For that matter, the particular importance of the waste products of any pure culture of _Clostridium_, in the formation of spores by that organism, has not to our knowledge been clearly elucidated.

**EXPERIMENTAL TECHNIQUE**

The strain of _C. sporogenes_ used was from the type culture collection of the Massachusetts Institute of Technology (*#82*), and had been carried aerobically for a year on an alkaline medium (containing 0.75 per cent agar, 0.2 per cent Difco nutrient broth, 0.1 per cent glucose, and 0.048 per cent NaOH) previously described by one of us (Williams, 1939b and c). About two months
before the experiments were initiated it was transferred to and carried anaerobically on a basal medium consisting of 1.53 per cent (3 strength: 1 per cent agar, 0.33 per cent peptone, 0.2 per cent meat extract), Difco nutrient agar, buffered to a pH of 6.7 with a 0.5 M NaH₂PO₄·H₂O to 0.5 M NaOH mixture. The buffer mixture was autoclaved separately, then mixed with one-ninth its volume of a four-to-six day old culture of the organism (previously heated to 80°C. for 10 minutes), and added to nine times its volume of basal medium, at a temperature of about 80°C. The buffer and medium were sterilized separately to prevent the precipitation of a large part of the calcium and magnesium content.

The cultures were grown in test-tubes containing 10 ml. of medium-buffer-inoculum mixture. These tubes were placed inside large Fischer desiccators, which were then partially evacuated, refilled with nitrogen, re-evacuated and filled with nitrogen; the nitrogen was passed in from commercial tanks through an anhydrous magnesium perchlorate tube and over heated copper coils, to remove impurities.¹ On the basis of the minimum pressure attained in the partially evacuated desiccator, it is estimated that a final atmosphere of about 5 mm. of oxygen and 755 mm. of nitrogen was obtained. The whole process was sufficiently speedy that the tubes were not yet solidified when the desiccator was placed in the 37°C. incubator.

The experiments were carried out on various modifications of the basal medium. The final pH and Eh of the medium were determined with the aid of the Beckman pH meter, Model G in a manner which one of us described in a previous study (Williams, 1939, a, b and c). The bottom half-inch of the culture-containing test-tube was filed around, cracked, and neatly broken off with a heavy spatula; the electrodes were immediately inserted almost to the bottom of the cylinder of culture medium. In the determination of the oxidation-reduction potential, a drift to a more negative potential was usually encountered in tubes containing growing cultures, and more reproducible Eh’s were obtained when

¹ This apparatus was loaned us by Mr. Ernest R. Barron, to whom we are very grateful.
at least fifteen minutes were allowed after introducing the electrodes.  

Smears were made as soon as the electrodes were removed; a heated nichrome loop was immersed into the bottom of the culture cylinder, a molten loopful quickly removed, and smeared over a circle of about $\frac{1}{4}$ inch radius on a glass slide. A second loopful was taken from a different portion of the culture cylinder, and was mixed thoroughly with the contents of the first loop. Control experiments showed that the exposure of these half-inch cylinders of medium, still inside the glass beakers, to air for a half hour or less after being cut, had little or no effect on the percent of spores formed. The smears were stained by the Shaeffer and Fulton modification of the Wirtz method, and examined within 48 hours.

The intensity of spore formation was determined by counting the number of free spores or sporangia (i.e. only those which retained the green stain) found among 100 cells. These enumerations coincided frequently in a remarkable fashion; usually, the same smear agreed within 2 to 5 per cent in duplicate counts, and separate smears from duplicate tubes agreed within 5 to 8 per cent. This degree of accuracy, it was felt, was sufficient for the need of the experiment. Duplicate tubes were prepared in all experiments in which spore counts were made and the averages are presented in the data below. In cultures older than 2 to 3 days, and in some media in much shorter periods, the extent of autolysis which the vegetative cells had undergone rendered the counts considerably less accurate.

**RESULTS**

*Experiment I. The effect of pH on spore formation by C. sporogenes*

The effect of additions of various mixtures of 0.5 M NaH$_2$PO$_4$. H$_2$O and 0.5 M NaOH to the unbuffered basal medium was first determined. The tubes were prepared in the manner described, except that no bacteria were inoculated. After 16 hours at room

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2 All oxidation-reduction potentials recorded are corrected for the potential of the calomel electrode by the addition of 0.246 volt to the observed reading.
temperature, they were removed from the anaerobic desiccator, and the pH and Eh of each tube was determined. The results are pictured in chart 1.

It must be emphasized that the oxidation-reduction potential readings of the sterile medium vary not only with the pH of the medium, but also with the extent of solidification (which in turn depends partly on the per cent of agar in the medium), and the temperature, at the moment when anaerobic conditions are ef-

![Chart 1](https://example.com/chart1.png)

**Chart 1. Relation between the oxidation-reduction potential of the medium and the per cent of spores formed, during the first eighty-nine hours of incubation at 37°C, represented at various hydrogen-ion concentrations**

fected. These variables were controlled as rigidly as possible during these experiments.

A series of tubes at different hydrogen ion concentrations were then inoculated, and the pH, Eh, and intensity of spore formation at the end of 9, 25, 36, 45, 69, and 89 hours of incubation at 37°C were determined. There was a slight tendency by the bacterial culture to alter the pH of the medium in some cases, to a pH more nearly neutral. Thus, tubes whose buffer mixtures were such that they should have shown a pH of 5.7, after three or four
days of incubation gave a pH of 5.95; and similarly an original pH of 8.65 was changed to 8.45. Such a tendency is often found in control (uninoculated) media, and may not be significant. In chart 1 the pH shown is that of the original culture medium.

No growth occurred at pH 5.3 in four days of incubation, but growth does take place between pH 5.7 and 8.75 (the alkaline limit of the experiment). Some sporulation occurs, in due time, wherever growth is initiated, but spores are not formed in large numbers (i.e. over 3 per cent) at or below pH 6.1, in four days. An optimum pH for spore formation lies between 6.9 and 7.4. At these pHs, the spores are first formed about 25 hours after inoculation, under the conditions of the experiment.

It may also be noted that in media whose original pH lay between 6.5 and 8.65 (i.e. between the pH limits which allowed considerable formation of spores) there is a correlation between the rate of formation of spores and the rise in Eh from its most negative peak. It seems to us extremely likely that the point of inflection of this reduction potential curve follows closely on the heels of the end of the logarithmic stage of the bacterial growth cycle, though the proof of this supposition is outside the scope of the present investigation. We wish therefore to present the hypothesis that the formation of spores by C. sporogenes accompanies the deaccelerating rate of proliferation of the bacterial culture, and that there may be causal relationship between these two phenomena. In this connection, it may be remembered that in 1928 Henrici showed that spores are formed by Bacillus megatherium at the close of the logarithmic stage of multiplication.

In chart 2 is represented the Eh of cultures at various hydrogen ion concentrations, before inoculation (Curve I) and after nine hours of incubation of inoculated media (Curve II). It may be seen that the difference between the Eh curves is greatest between pH 6.9 and 7.4. The rate at which the oxidation-reduction potential of a newly inoculated pure culture of an anaerobe begins to fall, in a medium of high initial Eh, may be considered a prime determinant of the speed with which the culture will begin active proliferation; therefore we may conclude the period of "lag" is also shortest between pH 6.9 and 7.4. Morrison and Rettger
have shown that the period of "dormancy" of (aerobic) spores is largely determined by conditions in the environment of the spores; the more favorable the medium, the more quickly will germination and growth be initiated. This principle may also be true of anaerobic bacteria. On the basis of this chain of assumptions, the conclusion may be drawn that the pH optima for spore formation by C. sporogenes (pH 6.9-7.4) coincide with the pH optima for the germination of spores and vegetative proliferation. Whether this would be a consequence of the "headstart" given the culture in a favorable medium, in the race to spore formation, or whether other factors may be of importance, has not yet been determined.

An attempt was also made in this experiment to determine the effect on spore formation of the exposure of proliferating cultures of C. sporogenes to air. A set of culture tubes (two at each pH) were removed from the anaerobic desiccator after 9 hours of incubation; the contents of these tubes had not yet solidified probably because the tubes were at a temperature of 80°C. when placed inside the thick walled desiccator, and contained only 1 per cent agar. They were then incubated aerobically for 26 hours more (i.e. a total of 35 hours of incubation) and the per cent of spores formed was then determined. When the results of these "aerobic" tubes were compared with those from the tubes in-

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**Chart 2. Variation of Eh with pH in uninoculated media (Curve I), and in inoculated media after nine hours of incubation (Curve II)**

- Oxidation-reduction potential (Eh) is plotted against pH.
- Curve I shows the variation of Eh with pH in uninoculated media.
- Curve II shows the variation of Eh in inoculated media after nine hours of incubation.
- The Eh values range from 0 to 0.5 volts, and the pH range is from 5.0 to 9.0.

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cubated anaerobically for 36 hours, some increase in the per cent of spores found in the media which had been exposed to air, was evident in the tubes of more acid pH (6.5 and 6.9). However, the differences were not so great that a definite conclusion could be drawn from them.

Experiment II. The effect on spore formation of variations in the peptone content of the medium

Difco bacto-peptone, meat extract, and bacto-agar were used in the preparation of media containing 1 per cent agar, 0.2 per cent meat extract, and varying concentrations of peptone. These were inoculated and buffered to produce a pH of about 6.65. One set of duplicate tubes was rendered anaerobic at about 85°C,

| TABLE 1 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | PER CENT OF PEPTONE IN MEDIUM | 0.003 | 0.1 | 0.2 | 0.33 | 0.5 | 1.0 | 2.0 | 3.0 | 5.0 |
| Final pH        | 6.65 | 6.65 | 6.65 | 6.65 | 6.65 | 6.7 | 6.7 | 6.8 | 7.0 |
| Per cent spores in medium rendered anaerobic at 40°C... | No growth | No | 0 | 0 | 1 | 3 | 7 | 35 | 50 |
| Per cent spores in medium rendered anaerobic at 85°C... | No growth | No | 2 | 19 | 31 | 39 | 47 | 40 | 38 | 33 |

and another set was cooled to 40°C before the Fischer desiccator was evacuated. The average results of duplicate tubes, read after 40 hours, are given in table 1.

Those tubes which were cooled to 40°C before the desiccator was evacuated exhibit a much less degree of growth and spore formation in the lower concentrations of peptone than in the higher concentrations. However, all these tubes containing peptone concentrations of 0.2 per cent or over exhibited considerable numbers of spores at the end of eight days of incubation. Apparently the period of lag had been lengthened by the air entrapped in the medium during the cooling process. In those media which contained large amounts of peptone, the reducing qualities of the peptone had overcome the oxidizing quality of
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the entrapped oxygen, and had lowered the Eh from the point to which the oxygen had raised it.

These results are cited to demonstrate the ease with which the false conclusion, that increasing peptone concentrations greatly accelerate the rate of formation of spores by C. sporogenes, might be drawn. In those tubes which were rendered anaerobic at 85°C., a much slighter stimulation is evident, when the concentration of peptone was enough to maintain good growth; in the highest concentrations the per cent of spores formed even falls off somewhat. These results are rendered less accurate, however, because of the extent to which the vegetative cells (and even spores) were autolyzed in the higher concentrations of peptone and because of the fact that the larger concentrations of peptone resulted in a slight rise in the final pH of the medium to a point more favorable for growth and sporulation. Notwithstanding, we may conclude that in media otherwise favorable, the increase of the concentration of peptone beyond a necessary minimum does not greatly affect the rate of formation of spores by C. sporogenes.

Experiment III. The effect on spore formation of variations in the meat extract content of the medium

Tubes containing 1 per cent agar, 0.33 per cent peptone, and varying concentrations of Difco meat extract were prepared, inoculated, and buffered to produce a pH of about 6.7. One set of duplicate tubes was about 85°C. in temperature when the desiccator was evacuated, and one was about 45°C. After 40 hours of incubation the per cent of spores formed was determined, the results being as shown in table 2.

It is evident that under anaerobic conditions the concentration of the indiscriminate mixture of bacterial foods which are combined in meat extract has even less influence on the rate of spore formation by C. sporogenes than has the concentration of peptone.

Experiment IV. The effect of carbohydrates on the formation of spores

To four series of tubes of basal medium were added glucose, maltose, lactose, and sucrose in one per cent concentrations; a
fifth set was used as a control. The tubes were rendered anaerobic when their temperature was about 60°C. The results are listed in table 3.

The data obtained with maltose were almost exactly the same as those found with glucose. It is obvious that the presence in the medium of either of these fermentable carbohydrates results in the inhibition of spore formation. Whether this is the result of the drop in pH, or of some other product of carbohydrate fer-

<table>
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<tr>
<th>TABLE 2</th>
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<tbody>
<tr>
<td><strong>PER CENT MEAT EXTRACT IN THE MEDIUM</strong></td>
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<tr>
<td><strong>Per cent spores in medium rendered anaerobic at 45°C...</strong></td>
</tr>
<tr>
<td>No growth</td>
</tr>
<tr>
<td><strong>Per cent spores in medium rendered anaerobic at 85°C...</strong></td>
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<table>
<thead>
<tr>
<th>TABLE 3</th>
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<tbody>
<tr>
<td><strong>CARBOHYDRATE PRESENT</strong></td>
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<tr>
<td><strong>TIME</strong></td>
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<tr>
<td><strong>pH</strong></td>
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<tr>
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<tr>
<td>44</td>
</tr>
<tr>
<td>50</td>
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</table>

mentation, cannot be determined from these data. However, the per cent of spores formed coincides with that formed in controlled buffered media of equivalent pH.

Sucrose gave results very much like those obtained in the control tubes. However, lactose for some unknown reason, apparently stimulates the formation of spores. Noguchi and Fitzgerald (1910) have reported a similar stimulation of spore formation by C. welchii by non-fermented sugars.
Experiment V. The interrelating effects on spore formation of variations in the concentrations of nutrients and of carbohydrates

In this experiment, the alkaline medium reported by one of us (Williams, 1939, b and c) for the aerobic growth of anaerobes was used. Four series of tubes were prepared, containing 0.75 per cent agar, 0.048 per cent NaOH, and:

Series 1. 0.1 per cent glucose and 0.2 per cent nutrient broth  
Series 2. 0.1 per cent glucose and 0.8 per cent nutrient broth  
Series 3. 1 per cent glucose and 0.2 per cent nutrient broth  
Series 4. 1 per cent glucose and 0.8 per cent nutrient broth

<table>
<thead>
<tr>
<th>NUTRIENT</th>
<th>GLUCOSE</th>
<th>TIME</th>
<th>FINAL pH</th>
<th>SPORES FORMED</th>
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<tbody>
<tr>
<td>per cent</td>
<td>per cent</td>
<td>hours</td>
<td></td>
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<tr>
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<td>0.1</td>
<td>41</td>
<td>6.85</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
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<tr>
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<td>69</td>
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<tr>
<td>0.8</td>
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<td>43</td>
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<tr>
<td>0.8</td>
<td>1.0</td>
<td>70</td>
<td>6.15</td>
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To tubes containing 9 ml. of these media were added 0.59 ml. of sterile 0.5 M NaH₂PO₄·H₂O, 0.31 ml. of sterile 0.5 M NaOH, and 0.1 ml. of a 4- to 6-day old culture of C. sporogenes on the basal medium previously described. One set of duplicate tubes of each series was grown aerobically, and one set was grown under an oxygen tension of about 5 mm.; there was, however, comparatively little difference between the final per cent of spores observed in these two sets. Usually those tubes grown aerobically showed a slightly higher percent of spores formed. The results from the tubes grown anaerobically are given in table 4.

It is evident that in media of low nutrient concentration, the
presence of 0.1 per cent or 1.0 per cent glucose inhibits the formation of spores of *C. sporogenes*. The possibility presents itself that this inhibition is due to some product of fermentation other than the lowering of the pH by the acid formed, since the pH is not reduced to the levels which inhibit sporulation.

On the other hand, a considerable percentage of spores are formed in media containing 0.8 per cent nutrient broth and 1.0 per cent glucose, despite a final pH as low as 6.1—a pH which we have already found limits spore formation greatly. In similar tubes to which no buffer was added, a final pH of 5.25 to 5.5 was attained in 70 hours, yet 6 per cent of the total number of cells present were spores. Perhaps the initial alkalinity of the medium contributed in these cases to the formation of spores. At any rate, we may conclude that the extent of inhibition of sporulation by the presence of fermentable carbohydrates depends considerably on the nutrient concentration of the medium.

**DISCUSSION OF HYPOTHESES**

One of the most unusual aspects of this problem of the significance and physiology of the bacterial spore is the ease with which reputable workers arrive at completely opposing results. For example, very different conclusions have been published concerning the chemical composition of spores (Leifson, 1931; Virtanen and Pulkki, 1933), their enzymatic activity (Cook, 1932; Virtanen and Pulkki, 1933) their antigenicity (Krauskopf and McCoy, 1937; Howie and Cruickshank, 1940), etc. These seemingly irreconcilable conclusions are frequently the result of differences in the experimental technique employed, but in many instances they are probably due to actual differences between species and between strains. The importance of the cultural background and past history of the strain cannot be overemphasized (Brunstetter and Magoon, 1932; Hoogerheide and Kocholaty, 1938). Thus, there is perhaps some justification for the large and varied assortment of reasons advanced as causes of spore formation.

Spores differ greatly from vegetative cells in many respects,—not only morphologically and in their very much enhanced powers...
of resistance, but also in their antigenic composition (in some cases), in the activity or availability of at least some of their enzyme systems (Cook, 1932; Virtanen and Pulkki, 1933), in their refractivity and other physical properties, etc. Possibly a complete reorientation of their molecular components occurs in the transition from one stage to another. Nevertheless, the impetus to pass from the vegetative cell to the spore, or vice versa, is probably not so complex a phenomenon. The "catalyst" which initiates the change, which sets in motion the complex colloidal, biochemical and cytological changes which accompany or result in the formation of spores,—this stimulus may make itself felt in a very few minutes (Fischoeder, 1909; Howie and Cruickshank, 1940), but the consummation of the process takes much longer. With this restriction, the process of transition from spore-form to vegetative-form is probably reversible, depending on the presence or absence of the "catalyst."

It is logical to believe that the processes of cell division and spore formation are incompatible (i.e. as far as the individual, not the bacterial community, is concerned). A considerable amount of energy is unquestionably involved in both these processes, and it is highly improbable that the forces within the bacterial cell would contribute sufficient energy to these diverse functions, simultaneously, to affect both of them. However, during a portion of the growth cycle (e.g. the logarithmic phase of growth) most or all of the cells in the culture are metabolizing, growing in volume, and consequently (Rashevsky, 1936) dividing at a rapid rate. Therefore, during this period it is unlikely that spores would be formed. It is only when the degree of metabolic activity of the individual cell becomes considerably less, that the tendency to form a spore may be strong. This is equivalent, in terms of the bacterial community, to the "stationary period" or peak of the growth curve, and probably follows immediately on the negative peak of the potential curve.

The question immediately presents itself, why does not spore formation always take place? Why do some "unfavorable" and some "favorable" conditions inhibit it in part or completely? There are several possible answers. In some cases, undoubtedly,
the rate of change from an environment suitable for a high degree of metabolic activity, to one far less favorable, is extremely rapid. In a rapidly growing community of aerobic bacteria, the oxygen deficiency may develop so quickly that the cells are rendered dormant, or killed; the period during which the cell feels "uncomfortable" but not yet "suffocated" is not long enough to allow the completion of the process of spore formation. Possibly the accumulation of extra-cellular enzymes in the medium results in a continued increase in metabolic wastes, at an accelerated rate, even after the cells have first responded to the effect of this toxic accumulation. The cells may be so injured in a very short time that they are incapable of completing the changes necessary for sporulation.

Other possibilities which present themselves include 
(a) greater hydrogen ion concentrations in some cases, or 
(b) some specific fermentation or metabolic products, may inhibit or interfere with a specific mechanism vital in the chain of events which results in spore formation; 
(c) carbohydrates or their fermentation products may reinduce the germination of spores as soon as they are formed; 
(d) spores may be autolyzed or dissolved in carbohydrate media soon after they are formed, under some conditions.

It is to be hoped that further investigation will throw light on some of these problems.

SUMMARY AND CONCLUSIONS

1. In a two-thirds strength Difco nutrient agar medium, buffered with phosphates, the optimum hydrogen-ion for sporulation of Clostridium sporogenes was between pH 6.9 and pH 7.4. Sporulation is inhibited at or below pH 6.1, almost completely, for at least the first four days of anaerobic incubation.

2. An increase in the concentration of peptone beyond a necessary minimum does not greatly affect the rate of formation of spores by C. sporogenes; variations in the meat extract content of the medium have even less influence, spores being formed in almost the complete absence of this ingredient.

3. The presence of 1 per cent fermentable sugar in dilute nutrient agar results in almost complete inhibition of spore for-
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mation. The presence of 1 per cent lactose stimulates the rate of sporulation.

4. In an originally alkaline medium, the presence of 1 per cent fermentable sugar inhibits sporulation only in media which contain small concentrations of available nitrogen.

5. The belief is expressed that spore formation is a natural phenomenon in the developmental cycle of C. sporogenes, and occurs unless the vegetative cells are prevented from forming them. Results indicate that there may be a fundamental relationship between the decrease in metabolic activity from its maximum, and the onset of sporulation.

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